Phase I Trial of a Melanoma Vaccine with $gp100_{280-288}$ Peptide and Tetanus Helper Peptide in Adjuvant: Immunologic and Clinical Outcomes[1]

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ABSTRACT

A melanoma vaccine composed of HLA-A2-restricted peptide YLEPGPVTA ($gp100_{280}$), with or without a modified T-helper epitope from tetanus toxoid AQYIKANSKFIGITEL, has been evaluated in a Phase I trial to assess safety and immunological response. The vaccines were administered s.c. in either of two adjuvants, Montanide ISA-51 or QS-21, to 22 patients with high-risk resected melanoma (stage IIB–IV). Local and systemic toxicities were mild and transient. We detected CTL responses to the $gp100_{280}$ peptide in peripheral blood in 14% of patients. Helper T-cell responses to the tetanus helper peptide were detected in 79% of patients and had a Th1 cytokine profile. One patient with a CTL response to gp100 had a recurrence in a lymph node 2 years later; her nodes contained $CD8^+$ cells reactive to $gp100_{280}$ (0.24%), which proliferated in response to peptide. The overall survival of patients is 75% (95% confidence interval, 57–94%) at 4.7 years follow-up, which compares favorably with expected survival. Four of 14 patients who completed at least six vaccines subsequently developed metastases, all of which were solitary and surgically resectable. They remain alive and clinically free of disease at last follow-up. Data from this trial demonstrate immunogenicity of the $gp100_{280}$ peptide and suggest that immune responses may persist long-term in some patients. The frequency and magnitude of the CTL response may be improved with more aggressive vaccination regimens. Although this Phase I study was not intended to evaluate clinical benefit, the excellent survival of patients on this protocol suggests the possibility of a benefit that should be assessed in future studies.

INTRODUCTION

Human melanoma cells express antigens recognized by CTLs. These include antigens created by unique, random mutations and those that are commonly expressed on melanomas from many different patients (1). The most widely shared melanoma antigens recognized by CTLs are derived from melanocyte differentiation proteins including tyrosinase, gp100, MART-1/Melan-A, and gp75. Over half a dozen peptides originating from gp100 have been defined in the HLA-A2 setting (2–5), and at least 4 of them are confirmed to be naturally processed and presented in melanoma (4).

The nonamer peptide YLEPGPVTA was defined as an epitope for CTLs from five different HLA-A2 patients with melanoma (2). This sequence represents residues 280–288 of the melanoma differentiation protein gp100 ($gp100_{280}$). It is unique in that it could be recognized by CTLs at very low concentrations, at or below the pM level (2). This peptide induces CTL responses *in vitro* in a substantial proportion of patients (6); thus, its immunogenicity has been suggested indirectly. When originally defined, it was identified among peptides eluted from HLA-A2.1 molecules from human melanoma cells when those naturally processed peptides were evaluated by mass spectrometry (2). Thus, $gp100_{280\ 288}$ is naturally processed and presented in this form. It appears to be presented at the cell surface at low copy number in HLA-A*0201^+ individuals; yet even at that low copy number its expression is adequate for CTL recognition and tumor cell lysis (2). Because this peptide is recognized by CTLs at low concentration in a significant number of melanoma patients in multiple studies, it is an appealing antigen to evaluate in a clinical tumor vaccine trial.

Vaccines that simultaneously induce helper T cells and CTLs may be more effective than those that induce CTLs only (7, 8). At the time this trial protocol was prepared, however, no class II MHC-restricted epitopes for helper T cells had been

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defined from melanoma proteins. Instead, a tetanus peptide was included in the trial as a nonspecific helper epitope, because virtually all Americans have been vaccinated against tetanus. The peptide QYIKANSKFIGITEL (p2), representing amino acid residues 830–844, has been shown to bind class II MHC molecules of all patients tested and specifically to HLA-DR1, DRw15 (2), DRw18 (3), DR4Dw4, DRw11 (5), DRw13(w6), DR7, DRw8, DR9, DRw52a, and DRw52b, which account for 80–90% of the population (9–11). The NH_2-terminal glutamine (Q) residue is highly susceptible to conversion to pyroglutamate. To stabilize the structure, we modified that peptide by addition of an NH_2-terminal alanine residue, and this modified tetanus peptide AQYIKANSKFIGITEL (Tet_{A830}) was included in the vaccine protocol. The ability of Tet_{A830} to induce T-helper responses is not abrogated by addition of one or several residues to the NH_2 terminus of the peptide (9–11). The patients were divided into three groups, who were vaccinated with: (*a*) the gp100 peptide alone; (*b*) the gp100 peptide plus the tetanus peptide; or (*c*) a fusion peptide incorporating both peptide sequences.

The optimal adjuvants for vaccinating with peptides had not been well-studied in humans. Thus, we used two different adjuvants for the vaccines in the trial. Montanide ISA-51 (Seppic, Inc.) is a mineral oil-based adjuvant analogous to incomplete Freund's adjuvant, which must be administered as an emulsion. QS-21 (Antigenics; Aquila Biopharmaceuticals, Framingham, MA) is a highly purified, water-soluble saponin that handles as an aqueous solution (12, 13). Both have been used in preclinical studies and in clinical trials and have the ability to induce CTL responses in animals and in humans (10, 14–17).

We have completed a Phase I trial of vaccination with the $gp100_{280\ 288}$ peptide YLEPGPVTA, with or without the modified T-helper epitope from tetanus toxoid, AQYIKANSKFIGITEL. Patients were also randomized to either of two different adjuvants Montanide ISA-51 or QS-21. In the present study, we report on safety, immunogenicity, and clinical outcome with this vaccine approach.

PATIENTS AND METHODS

Patients. Patients with resected stage IIB, III, and IV melanoma or patients with minimal metastatic disease were eligible. Entry criteria included ages 18–79, expression of HLA-A2 on peripheral blood mononuclear cells, and expression of gp100 by melanoma cells based on immunohistochemistry. Patients were not eligible if they were pregnant; had received cytotoxic chemotherapy, steroids, IFNs, or other investigational drugs within the preceding 3 months; or if they had ever received a melanoma vaccination. Eligible patients were studied with informed consent and with Institutional Review Board and FDA[4] approval under IND 6453: Phase I Protocol for the Evaluation of the Safety and Immunogenicity of Vaccination with a Synthetic Melanoma Peptide in Patients with High Risk Melanoma.

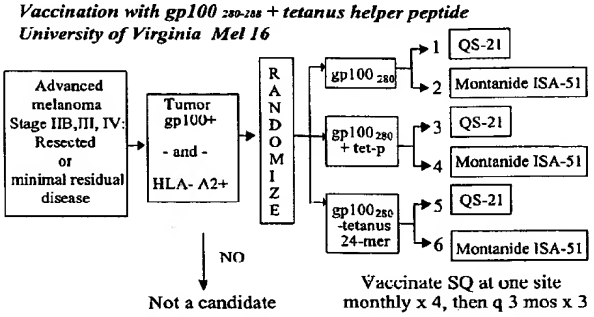

Fig. 1 Flow diagram for Mel16 peptide vaccine trial. Patients were randomized first to groups 1 and 2, and then subsequent patients were randomized to groups 3–6.

Peptide Vaccine Preparations. The peptide vaccine preparations used for this trial included peptide $gp100_{280}$ (YLEPGPVTA), tetanus peptide (AQYIKANSKFIGITEL), and fusion peptide gp100-tet (YLEPGPVTAQYIKANSKFIGITEL). Peptides were synthesized at the UVA Biomolecular Core Facility with a free amide NH_2 terminus and free acid COOH terminus. Each was provided as a lyophilized peptide, which was then reconstituted in sterile water and diluted with Lactated Ringer's solution (LR, Baxter Healthcare, Deerfield, IL) as a buffer for a final concentration of 67–80% Lactated Ringer's in water. These solutions were then sterile-filtered, placed in borosilicate glass vials, and submitted to a series of quality assurance studies including confirmation of identity, sterility, general safety, and purity, in accordance with FDA guidelines, as defined in IND 6453. Tests of peptide stability demonstrated no decrease in purity or in the peptide concentration, when these peptide solutions were stored at −20°C ~3 years.

QS-21 and Montanide ISA-51 adjuvants were provided by Antigenics (Aquila Biopharmaceuticals) and Seppic, Inc. (Paris, France), respectively, in sterile, single-use vials.

Immunization Protocol. Patients received a vaccine containing 100 μg of the HLA-A2-restricted melanoma peptide YLEPGPVTA ($gp100_{280\ 288}$), with or without the HLA-DR-restricted tetanus helper peptide AQYIKANSKFIGITEL. The patients were treated in six groups (Fig. 1). Groups 1 and 2 were vaccinated with 100 μg of the gp100 peptide in adjuvant, alone. Groups 3 and 4 were vaccinated with 100 μg of the gp100 peptide plus 190 μg of the tetanus helper peptide. The higher dose of the tetanus peptide was calculated to provide equimolar quantities of the helper and cytotoxic epitopes. Groups 5 and 6 were vaccinated with a 24-mer peptide comprising the amino acid sequences of both the gp100 peptide and the tetanus peptide (YLEPGPVTAQYIKANSKFIGITEL). The first 9 patients were randomized between groups 1 and 2, and the subsequent 13 patients were randomized among groups 3–6. The peptides, in 1-ml aqueous solution, were administered either as a solution/suspension with 100 μg of QS-21 (groups 1, 3, and 5) or as an emulsion with 1 ml of Montanide ISA-51 adjuvant (groups 2, 4, and 6). Patients were immunized at day 0 and months 1, 2, 3, 6,

[4] The abbreviations used are: FDA, Food and Drug Administration; ELISPOT, enzyme-linked immunospot; IL, interleukin; PBL, peripheral blood lymphocyte; DTH, delayed-type hypersensitivity; SI, stimulation index; TCR, T-cell receptor; CI, confidence interval.

9, and 12, with the peptides plus adjuvant, for a total of seven immunizations. With rare exceptions, the vaccinations were administered to the same arm with each vaccine. They were administered s.c. A schematic of the protocol is presented in Fig. 1.

The patients did not receive any concomitant treatments during the course of vaccination. In particular, none of the patients received IFN-α during the course of the vaccinations. IFN was not approved for the adjuvant therapy of melanoma when this study was initiated.

Stopping rules were written such that patients were first entered into groups 1 and 2, with the gp100 peptide plus adjuvant alone, and accrual into groups 3–6 (tetanus peptide added) was begun only after safety was determined for patients in groups 1 and 2. Thus, patients were randomized to groups 1 and 2 initially, and then remaining patients were randomized to groups 3–6. The stopping rule was based on considering the maximum tolerated dose to have been exceeded if two patients of six experienced toxicity of grade 3 or greater. Had that occurred, the trial was to be stopped, with the possibility of considering a revised protocol using a lower dose of antigen or adjuvant.

Cell Lines Used. T2 is a mutant human T/B-cell hybrid that lacks the transporter associated with antigen processing (TAP) but expresses HLA-A*0201 (18). It was provided by Peter Cresswell (Yale University, New Haven, CT). HLA typing was performed by microcytotoxicity assay on autologous lymphocytes (One Lambda, Canoga Park, CA).

Peptides. Class I MHC-associated peptides used in the laboratory studies included YLEPGPVTA ($gp100_{280\ 288}$; Ref. 2), YMDGTMSQV ($tyrosinase_{368\ 376D}$; Ref. 19), ALLAVGATK ($gp100_{17\ 25}$; Ref. 20), and YLKKIKNSL (Malaria $CSP_{334\ 342}$; Ref. 21), plus the peptides used for vaccination as described above.

ELISPOT Assays. Lymphocytes were cultured in complete RPMI 1640 with 10% heat-inactivated human AB serum (Sigma Chemical Co., St. Louis, MO), 2 mM L-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin (Pen-Strept; Life Technologies, Inc., Grand Island, NY). They were assayed 2 weeks after one or two sensitizations *in vitro* with peptide. For these sensitizations, 2×10^6 lymphocytes/ml in complete medium were incubated with synthetic peptide (40 μg/ml) for 2 h at 37°C, 5% CO_2. The cells were pelleted, resuspended in complete medium containing IL-2 (20 units/ml; Chiron Corp., Emeryville, CA), and cultured in 24-well plates (Linbro; ICN Biomedicals, Inc., Aurora, OH) starting at $3–4 \times 10^6$ cells/well. Complete medium with IL-2 was replaced as needed. When two *in vitro* sensitizations were performed, the second sensitization used autologous irradiated PBLs pulsed with peptide as stimulator cells at a 1:3 stimulator:lymphocyte ratio, which were added to the cultures on day 7. Cells were cultured a total of 14 days prior to evaluation by ELISPOT assay. Nonstimulated lymphocytes were tested in IL-10 ELISPOT assay without prior sensitizations.

Immulon 2 flat-bottomed plates (Dynatech, Chantilly, VA) were coated with anti-IFN-γ monoclonal antibodies (M-700A; Endogen, Woburn, MA) or anti-IL-10 monoclonal antibodies (M-010; Endogen). For IFN-γ assays, lymphocytes were mixed with equal numbers of antigen-presenting cells (T2, C1R-A1, and C1R-A3, depending on HLA type) alone or cells pulsed with peptide (40 μg/ml) in the first row of the plate. Serial dilutions were made, such that responder cell number ranged from 100,000 to 5,000 per well. For IL-10 assays, peptides were added (10 μg/ml) directly to mononuclear cells, without added antigen-presenting cells. Plates were incubated at 37°C, 5% CO_2 for 18 h. After extensive washing with 0.025% Tween 20 in water, plates were incubated with a biotin-labeled secondary antibody to IFN-γ (M-701B; Endogen) or IL-10 (M-011-B; Endogen), then washed again, and incubated with avidin conjugated with alkaline phosphatase (13043E; PharMingen, San Diego, CA). After washing, plates were developed with the 5-bromo-4-chloro-3-indolyl phosphate substrate in 1% low melting agarose. The number of blue spots corresponding to the number of cells secreting IFN-γ (or IL-10) were counted in each well, visually, using a Nikon TMS microscope with ×2 objective. Each sample was tested in triplicate at each of several dilutions of lymphocytes. The average number of spots produced by lymphocytes incubated with cells alone was compared with that produced by lymphocytes incubated with cells loaded with peptide. The frequency of T-cells reactive to peptide was calculated based on this difference.

ELISPOT assays were performed on the prevaccine blood sample, and representative samples were taken after each vaccine, ranging from 3 to 7 test dates/patient. Twenty-one of the 22 patients were evaluated in this manner for IFN-γ, with 1 patient excluded because of his very early withdrawal from the study.

IFN-γ Release Assays. In selected cases, CTL responses detected by ELISPOT were confirmed in a second assay to measure responses to peptide antigen. T cells sensitized with peptide *in vitro*, as above, were incubated with stimulator cells at a responder:stimulator ratio of 2:1 (2×10^5 CTLs and 1×10^5 target cells/well in a 96-well plate), in assay medium for 24–48 h. IFN-γ released in the medium was quantitated by ELISA using the matching anti-IFN-γ antibody pair M-700A and M-701B (Endogen) in accordance with the manufacturer's directions. Human recombinant IFN-γ (Life Technologies, Inc.) was used for calibration.

DTH Responses and Recall Antigen Responses. Patients received injections at separate sites intradermally with each of four standard recall antigens: Trichophyton (Hollister Stier, Spokane, WA); Tetanus (Wyeth-Ayerst Laboratory, Philadelphia, PA); Candida (Hollister Stier); purified protein derivative (PPD, Pasteur Merieux Connaught, Swiftwater, PA), and with 10 μg of the gp100 peptide, without adjuvant, prior to the first vaccine, and the induration at each skin test site was recorded at 24 and 48 h. This was repeated 6 or 12 months after the first vaccine. A positive response is considered an area of induration 10 mm or greater in diameter.

Proliferation Assay. PBL samples collected before immunization, after three and after six immunizations, were cultured at 2×10^5/well in the complete medium with 10% human AB serum: (*a*) without any additional antigen; (*b*) in the presence of bovine albumin (0.5 μg/ml; Sigma Chemical Co., St. Louis, MO); (*c*) with tetanus toxoid (1:2000 dilution of vaccine prep; Wyeth-Ayerst Laboratories); or (*d*) with tetanus helper peptide Tet_{A830} (10 μM). Cells were plated in triplicate or quadruplicate per condition. On day 5, cells were labeled with

[^{3}H]thymidine (1 μCi/well; ICN, Costa Mesa, CA) for 8 h and then harvested and evaluated for [^{3}H]thymidine incorporation. Peptide-specific proliferation was determined as a difference between [^{3}H]thymidine incorporation in experimental wells and the highest negative control (medium or bovine albumin). We defined a SI as a ratio of maximal [^{3}H]thymidine incorporation in response to $Tet_{\Delta 830}$ during immunization to the preimmunization value for the response.

Cytokine Release Assay. PBL samples collected after three or six immunizations were depleted of $CD8^+$ cells using MACS MS (Miltenyi Biotec, Auburn, CA) or StemCell (Vancouver, British Columbia, Canada) separation columns. CD8-depleted cells (CD4-enriched) were cultured at 2×10^5/well in the complete medium with 10% human AB serum: (*a*) without any additional antigen; (*b*) in the presence of bovine albumin (0.5 μg/ml); (*c*) with tetanus toxoid (1:2000 dilution of vaccine prep; Wyeth-Ayerst Laboratories); or (*d*) with tetanus helper peptide $Tet_{\Delta 830}$ (10 μM). Cells were plated in duplicate per condition/time point. Supernatants were collected daily, days 1–5. Concentrations of IFN-γ, IL-4, and IL-10 in the supernatants were determined by ELISA using the matching antibody pairs from Endogen in accordance with the manufacturers' directions.

Peptide-MHC Tetramer Staining. Three peptide-MHC tetramer preparations (HLA-A*0201/YMDGTMSQV, HLA-A*0201/YLEPGPVTA, and HLA-A*0301/ALLAVGATK) were provided by National Institute of Allergy and Infectious Diseases MHC Tetramer Core Facility (Atlanta, GA). The specificity of tetramers was confirmed by titration using polyclonal CTL lines with known specificities (data not shown). Patient samples were evaluated after enrichment for $CD8^+$ cells using immunomagnetic labeling, followed by negative selection on separation columns (StemCell Technologies). Cells were stained with the phycoerythrin-labeled tetramers (1:100 dilution; 10 μg/ml), anti-CD8 antibody (APC, MHCD 0804, Caltag), and anti-TCR α/β chain antibody (FITC, 347773; Becton Dickinson, San Jose, CA). We used fluorescence-activated cell sorter Calibur (Becton Dickinson) with CELLQuest software to enumerate $tetramer^+/TCR^+/CD8^+$ cells. A tumor-free lymph node sample BRC2421 from a breast cancer patient was used as a negative control. The anti-TCR α/β antibody used has been shown not to block tetramer binding (22), and our initial assay optimization studies confirmed that (data not shown).

RESULTS

Patient Accrual. Twenty-two patients were accrued in this trial in the six groups shown in Fig. 1. Most of the patients (n = 15; 68%) had stage III disease, including 13 with nodal metastases and 2 with intransit metastases only. Among the 13 patients with node-positive disease, only 5 had disease limited to sentinel nodes. The remainder presented with gross palpable nodal disease, with 1–8 nodes positive (mean, 3.6; median, 2; 8, 7, 6, 2, 2, 2, 1, 1). Three patients (14%) had stage IIB disease, with primary melanomas >4 mm thick and negative nodes (2 with negative sentinel node biopsy and 1 with negative clinical exam only). Four patients (18%) had stage IV disease, including 1 with a resected lung metastasis and 3 with resected distant skin metastases, 2 from unknown primary lesions. One patient with stage III disease was entered into the protocol with small volume disease consisting of satellite metastases, but the remaining 21 patients were clinically free of disease at the time of study entry (Table 1).

Table 1 Patient characteristics

	n	%
Stage		
IIB	3	14
III	15	68
IV	4	18
Age (yr)		
≤50	11	50
>50	11	50
Gender		
Male	12	54
Female	10	46
Sites of metastasis		
None (sentinel node negative, 2; sentinel node not done, 1)	3	14
Nodes	13	59
Nonpalpable lymph node metastases (N1a, 4; N2a, 1)	5	(23)
Palpable lymph node metastases	8	(36)
N2b	2[a]	
N3	6[b]	
Intransit skin	2	9
Distant	4	18
Lung	1	
Distant skin (2 with unknown primaries)	3	
Prior IFN-α therapy		
No	14	77
Failed on IFN-α	3	14
Did not tolerate IFN-α	1	5
Took 1 yr of IFN-α without failure	1	5
No. of patients accrued in groups		
Group 1	4	18
Group 2	5	23
Group 3	4	18
Group 4	2	9
Group 5	4	18
Group 6	3	14
Adjuvant		
QS-21 (groups 1, 3, and 5)	12	54
Montanide ISA-51 (groups 2, 4, and 6)	10	45
Peptide		
$gp100_{280}$	9	41
$gp100_{280}$ + tetP	6	27
Chimeric molecule	7	32

[a] Includes 1 patient with recurrent nodal disease.
[b] Includes one patient with clinically evident disease at the time of protocol entry, in the form of multiple satellite metastases after resection of a palpable node.

Eighteen patients (82%) were entered on the protocol within 4 months of their last surgical procedure, 16 within 3 months. The others started 5, 9, 10, and 25 months after definitive surgery because of an interval trial of IFN-α (patients 1103 and 1108) or because of patient delay to consider options (patients 1117 and 1119). Five of the patients had had prior high dose IFN-α, three of whom progressed on or soon after IFN-α therapy (patients 1102, 1111, and 1118), and two who entered this trial after completing <1 month of high-dose IFN-α (1103) or a full-year of IFN-α at 3 million units/day off protocol (patient 1108). Otherwise, patients had not been treated with other therapy. Twelve patients completed the protocol's seven injections. Three patients completed six of the seven injections:

Table 2 Patients enrolled in protocol, with clinical outcome

Patient ID	Gender	Group	Stage	No. of vaccines	Reason off protocol	Time to progression (yr)	Site of progression	Follow-up[a] (yr)	At last follow-up	
									Alive?	NED?
1101	M	1	III	4	Progression	0.5	Multidistant	0.6	N	N
1102	M	1	III	7				4.6	Y	Y
1103	F	2	IIB	7				4.5	Y	Y
1104	F	2	IV	7		1.7	Skin	4.6	Y	Y
1105	F	1	IIB	7		2.5	Nodes	4.4	Y	Y
1106	F	2	III	7				4.1	Y	Y
1107	M	1	III	2	Progression	0.2	Intransit	1.6	N	N
1108	M	2	III	7				3.6	Y	Y
1109	M	2	III[b]	2	Progression	0.2	Lung	0.4	N	N
1110	M	3	III	7		1.8	Nodes	3.0	Y	Y
1111	F	3	III	6	Other cancer			0.9	Y	Y
1112	M	6	III	7				3.1	Y	Y
1113	M	5	III	6	Noncompliance			2.6	Y	Y
1114	M	4	IV	4	Progression	0.4	Nodes	1.2	N	N
1115	M	6	III	4	Progr or MI[c]	0.5	Not evaluable	0.5	N	N
1116	F	6	III	3	Progression	0.3	Intransit	0.9	Y	N
1117	M	3	III	7				2.4	Y	Y
1118	M	5	IV	7				2.1	Y	Y
1119	F	5	IV	7		1.5	Liver	1.9	Y	Y
1120	F	4	IIB	7				1.6	Y	Y
1121	F	5	III	4	Progression	0.5	Intransit	1.4	Y	N
1122	F	3	III	6	Adverse event			1.5	Y	Y

[a] Mean follow-up, 2.3 years; median follow-up, 2.0 years; median follow-up of living patients, 2.6 years. NED, no evidence of disease.
[b] This patient had minimal measurable disease at protocol entry.
[c] Progr or MI, progression or myocardial infarction.

1 was removed for toxicity after injection 6 (patient 1122); 1 patient failed to report for his last injection (patient 1113); and 1 because of progression of an unrelated cancer (breast cancer) requiring cytotoxic chemotherapy (patient 1111). Patients 1113 and 1122 have returned for follow-up since that time, without evidence of recurrence or of toxic sequelae. Seven patients were taken off the protocol because of tumor progression after two to four injections. All patients were followed for clinical outcome, and all patients were assessed for immunological responses except for 1, who was taken off the protocol for tumor progression after two injections. Additional clinical details are provided in Table 2.

Toxicity and Adverse Experiences. Most patients experienced mild and transient pain at the vaccine injection sites, but inflammation at those sites was mild enough that by the time of each subsequent vaccine, induration was usually absent. Patients often experienced very mild flu-like symptoms for ~1 day after each vaccine, but that usually occurred several days after the vaccine was administered. Individual toxicities are reported, based on NIH Common Toxicity Criteria, in Table 3. Mild toxicity was reported in 9 of 13 patients (69%) vaccinated with the gp100 peptides plus the tetanus helper peptide (groups 3–6), compared with 7 of 9 patients (77%) vaccinated with the gp100 peptide alone (P = not significant; data not shown). These results are summarized in Table 4.

Both adjuvants were very well tolerated with minimal toxicity, but there were more toxicities reported with QS-21 than with Montanide ISA-51 (Tables 3 and 4). Local toxicities were observed in 9 of 12 patients vaccinated with QS-21 (75%) and in 3 of 10 patients with Montanide ISA-51 (30%). The local toxicities observed with QS-21 vaccines were generally limited to local pain at the time of vaccine injection and were of very short duration. By χ^2 analysis, with correction for degree of freedom = 1, the difference in local toxicities between adjuvants does not reach significance ($\chi^2 = 2.81$; $P \sim 0.09$); however, this small sample size is not adequate to rule out differences between these two groups. Overall toxicities were higher with QS-21 as well, with one or more toxicities reported in 12 of 12 patients (100%), compared with 4 of 10 patients (40%) with Montanide ISA-51. By χ^2 analysis, with correction for degree of freedom = 1, this is significant ($\chi^2 = 7.09$; $P < 0.01$). Despite these differences, the vast majority of toxicities were limited to grade 1 and 2 toxicities, and all were transient.

One patient (no. 1122) had a transient grade 3 adverse experience after vaccination. This 23-year-old woman had received five vaccines without any adverse reaction. However, within 20 min after her sixth vaccine, she complained of pain in her right arm at the injection site (grade 2), numbness (grade 2), dizziness (grade 1), faintness, nausea (grade 2), stomach cramping (grade 2), and substernal tightness consistent with esophageal spasm (grade 3). She was assessed by the principal investigator (C. S.) and a research nurse practitioner (P. N.). She was hemodynamically stable, and oxygen saturation was 99% on room air. Physical exam was unremarkable. Her reaction was considered most consistent with an allergy, with a component of esophageal spasm. Thus, she was administered an oral antihistamine (Claritin). Her symptoms resolved within 90 min. Because this reaction raised a question of allergy to the vaccine, she did not receive a seventh and final vaccine. This was the last patient accrued into the study and was the only patient to experience a grade 3 toxicity. She was in group 3 (gp100 + QS-21 + tetanus peptide). A total of 8 patients received the

Table 3 Toxicities observed with each adjuvant, by grade

	QS-21				Montanide ISA-51				Total				
	None	1	2	3	None	1	2	3	None	1	2	3	%
Local skin, especially pain	3	7	2		7	3			10	10	2		55
Skin, other	8	4			10				18	4			18
Flu-like symptoms	10	2			7	2	1		17	4	1		22
Headache	9	3			9	1			18	4			18
Other pain	10	2			10				20	2			9
Stomatitis	12				9	1			21	1			5
Esophageal spasm	11			1	10				21			1	5
Other gastrointestinal	10	1	1		9	1			19	2	1		14
Hematological	11	1			10				21	1			5
Syncope	11	1			10				21	1			5
Other neurological	11		1		9	1			20	1	1		9
Dyspnea	12				9	1			21	1			5
Endocrine	12				9		1		21		1		5
Urinary	12				9	1			21	1			5
Any	0	9	2	1	6	3	1		6	12	3	1	73

Table 4 Summary table of maximum grade toxicity per patient, by vaccine group and by adjuvant

	Maximum grade toxicity					
	0	1	2	3	4	5
Groups 1 and 2	2	6	1	0	0	0
Groups 3–6	4	6	2	1	0	0
QS-21	0	9	2	1	0	0
Montanide ISA-51	6	3	1	0	0	0
Total	6	12	3	1	0	0

gp100 peptide with QS-21 (group 1+3), and 6 patients received the mixture of gp100 and tetanus peptide (group 3+4), with only this one transient grade 3 toxicity. Thus, the maximal tolerated dose was not reached for any of the components with the regimens used.

Four patients died with melanoma progression after completing this protocol or after being taken off the protocol for disease progression (Table 2). One patient developed tumor progression and neurological symptoms and lethargy associated with tumor progression (patient 1114), and 1 developed progression of a different cancer (breast cancer, patient 1111). These are not included in the toxicity listings above. In addition, one patient died while on this protocol. He developed symptoms suspicious for tumor progression, with marked lactate dehydrogenase elevation (~1500), and was scheduled for restaging studies and, because of known cardiac disease, for a cardiology evaluation within 3 days, but he died before those studies could be performed, probably because of either a myocardial infarction or tumor progression. This death was reported to the FDA because he died while enrolled in the protocol; however, his death occurred ~3 months after his fourth injection; therefore, it was not related temporally to any of his vaccines and is considered treatment unrelated and is not included in the toxicity assessment above. His disease was unevaluable but is considered a failure in terms of disease progression and mortality.

One additional patient, 1105, tolerated the vaccine protocol well but developed cutaneous lupus erythematosis almost 2 years after completing her vaccines. This was diagnosed by a skin biopsy and positive antinuclear antigen titer, and this was temporally associated with nodal recurrence of her melanoma close to the site of her skin changes. The lupus has not progressed and has required no therapy. No other autoimmune disease been observed in follow-up of these patients, except that 2 patients developed mild depigmentary skin changes during the protocol.

Because retinal pigment epithelium contains gp100, we evaluated specifically whether there was any ocular toxicity, by measuring visual acuity and performing opthalmoscopic exams at each visit, and by performing complete fundoscopic evaluation by an ophthalmologist including a fluorescein retinal angiogram. There was no evidence of visual defect or of loss of pigmented retinal epithelium on any of these patients.

CTL Responses to Vaccination with $gp100_{280}$ Peptide. Blood was collected at study entry and prior to each vaccine. Thus, blood was available 1 month after each of the first three injections and 3 months after each of subsequent injections. In multiple ELISPOT assays, we found no detectable CTL responses to the vaccinating peptide when the PBLs were assayed directly without *ex vivo* culture. We increased sensitivity of the assays by sensitizing the PBLs with $gp100_{280}$ peptide *in vitro*. In early studies, we sensitized with peptide once on day 0 and then evaluated T-cell response after 14 days in culture but found that more reliable results could be obtained with an ELISPOT for IFN-γ-secreting $CD8^+$ cells, performed on cryopreserved lymphocytes sensitized twice with peptide $gp100_{280}$ and then assayed on day 14. Patients were considered to have evidence of a T-cell response if reactivity to the immunizing peptide was greater than the response to both of two negative controls and was at least 4-fold greater than the prevaccine reactivity. Responses to the $gp100_{280}$ peptide were observed in 3 of 21 evaluated patients (14%), one each from group 1 (patient 1105), group 2 (patient 1108), and group 4 (patient 1120). Examples of data for two patients are shown in Fig. 2, *A* and *B*. For these 2 patients, responses were also evident after a single *in vitro* sensitization (data not shown). Assays for IFN-γ secretion corroborate ELISPOT findings as shown in Fig. 2, *C* and *D*. These

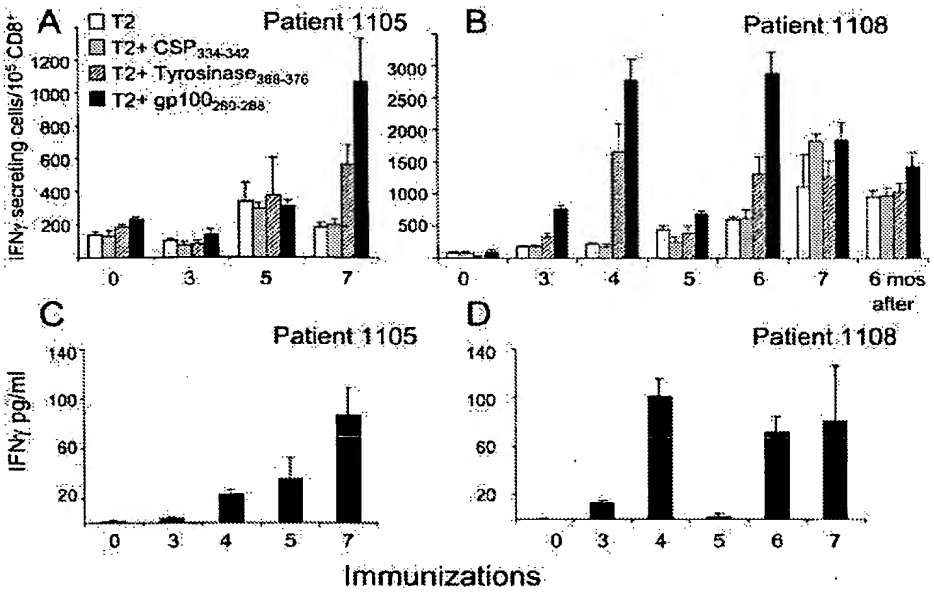

Fig. 2 Detection of CTL responses to vaccination. PBLs were collected prior to vaccination and 1 or 3 months after each vaccination and were cryopreserved. Subsequently, PBLs from each of several representative dates were cultured in parallel, with weekly sensitizations with the vaccinating peptide, $gp100_{280}$ (YLEPGPVTA) × 2. The first stimulation used peptide alone; the second stimulation used peptide presented on autologous PBLs. After 14 days from the initiation of culture, $CD8^+$ cells represented about 20–25% of the cells. $CD8^+$ cells were then purified by negative selection on StemCell columns and were evaluated in ELISPOT assays for reactivity to the gp100 peptide pulsed on T2 cells. Results from patient 1105 are shown in *A* and from patient 1108 in *B*. aliquots were also evaluated for secretion of IFN-γ, measured by ELISA assay in 24-h supernatants. IFN-γ secretion by PBLs in response to T2 + $gp100_{280}$ is shown for patients 1105 (*C*) and 1108 (*D*), with background reactivity to T2 alone subtracted. Background reactivity to T2 ranged from 7.7 to 53.7 pg/ml (mean, 21.6) for patient 1105 and from 2 to 24 pg/ml (mean, 3.9) for patient 1108. *Bars,* SD.

data support the conclusion that the peptide YLEPGPVTA is immunogenic *in vivo* in humans, but that the immune response detected in the peripheral blood is not striking either in its magnitude or its frequency.

CTL Recognizing $gp100_{280}$ Accumulated in Lymph Nodes 2 Years after Vaccination. One of these patients with a T-cell response evident in the PBLs (patient 1105) developed tumor progression in a regional inguinal node 1.5 years after completing this vaccine protocol. She underwent surgery (by C. S.) to remove the two tumor-involved nodes and the remainder of nodes in the inguinal nodal basin. She has since done well for ~2 additional years after surgery and remains clinically free of disease. Thus, this was an isolated nodal recurrence after vaccination.

The $CD8^+$ T cells infiltrating this tumor deposit were evaluated directly *ex vivo* (without culture or antigen exposure) using MHC-tetramers for HLA-A2/YLEPGPVTA. CTL recognizing YLEPGPVTA were identified as 0.24% (1 of 417) of the $CD8^+$ cells in that node (Fig. 3*A*). CTLs reactive to another A2-restricted melanoma peptide (YMDGTMSQV from tyrosinase), with which this patient was not vaccinated, were not observed. CTL recognizing the gp100 peptide were evident also in a tumor-free node from the same nodal basin but at lower frequency (Fig. 3*A*).

CTLs Recognizing $gp100_{280}$ in Tumor Metastasis Fail to Secrete IFN-γ in Response to Peptide *ex Vivo*. Lymphocytes from the metastatic tumor-involved node were evaluated by ELISPOT for their ability to secrete IFN-γ in response to the peptide $gp100_{280}$. No IFN-γ-secreting cells were identified above background (Fig. 4*B*). However, there was high spontaneous release of IL-10 among lymphocytes from peripheral blood (~1:500) at the time of the patient's lupus diagnosis and tumor recurrence, and there was also spontaneous nonspecific release of IL-10 by cells in her tumor-involved node (Fig. 4*A*).

To determine whether the lymphocytes in the tumor-involved node could be activated *in vitro* by the autologous metastatic melanoma cells, lymphocytes and tumor cells from the tumor-involved node were cocultured in IL-2 (20 units/ml)-containing medium for 14 days and then evaluated by ELISPOT for reactivity to the gp100 peptide. This approach has been effective in a prior study for detection of CTL responses to an HLA-A3-restricted gp100 peptide (23). However, in the present analysis of this patient's tumor, no YLEPGPVTA-specific cells were found, suggesting either a failure of the metastatic tumor to present the gp100 peptide or a tolerizing effect of the tumor on YLEPGPVTA tetramer-positive T cells (data not shown). The original primary tumor was diffusely positive for gp100 expression and tyrosinase expression by immunohistochemistry, but the metastatic tumor cells in the inguinal node were positive for

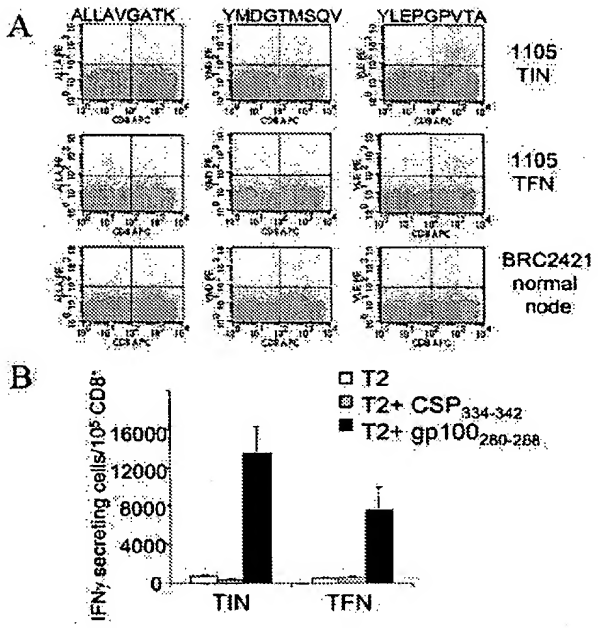

Fig. 3 Peptide-reactive CTLs identified in tumor deposit 1.5 years after vaccination. *A,* T cells infiltrating tumor of patient 1105 and T cells from a negative node were evaluated directly *ex vivo,* after enrichment for $CD8^+$ cells by negative selection on a StemCell column, and were tested with MHC tetramers HLA-A2/YMDGTMSQV, HLA-A2/YLEPGPVTA, and HLA-A3/ALLAVGATK. A normal node from a patient with breast cancer was used as a negative control. HLA-A2/$YLEPGPVTA^+$ cells are 0.24% of $CD8^+$ cells in the tumor-involved node (*TIN*) and 0.08% in the tumor-free node (*TFN*), with all negative controls being 0.02–0.04%. *B,* lymphocytes infiltrating a tumor-involved node from patient 1105, 1.5 years after completing vaccination, were stimulated with the immunizing peptide (gp100) weekly × 2. $CD8^+$ cells were selected and then evaluated by ELISPOT at 14 days. *Bars,* SD.

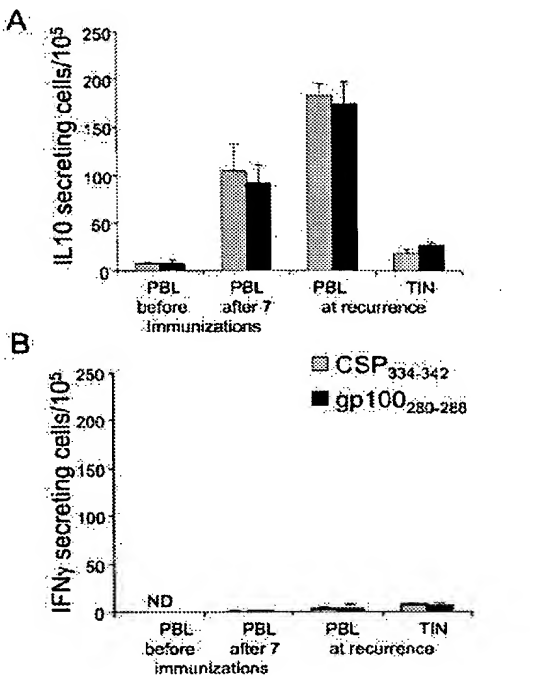

Fig. 4 *Ex vivo* analysis of patient 1105's tumor-infiltrating lymphocytes and PBLs for secretion of IFN-γ or IL-10. A cryopreserved specimen from the tumor-involved node and PBLs of patient 1105 were thawed and then evaluated in two parallel ELISPOT assays, one with a readout of IFN-γ and another for enumeration of IL-10-secreting cells. Quantitation was performed as described. We tested the prevaccination PBL sample, PBLs collected 6 months after the last vaccine, and the PBL sample collected 1 year later, at 1 week after the patient presented with a rash and enlarged inguinal node. *TIN* data represent tumor-involved node from a surgical resection of inguinal nodes. *Bars,* 1 SD of triplicate wells. *ND,* not done because of a limited sample.

gp100 expression in only 50% of cells, and the expression was weak, whereas expression of tyrosinase continued to be evident in virtually all cells, many at moderate to strong intensity (not shown).

However, $CD8^+$ T cells producing IFN-γ in response to the $gp100_{280}$ peptide were detected in this tumor-involved node after two *in vitro* sensitizations with peptide (Fig. 3*B*). In addition, gp100-reactive T cells were similarly evident in a lymph node in the same inguinal basin, which was not involved with tumor. The magnitude of the response is more than 10 times higher than that observed in the postvaccination blood sample after a comparable *in vitro* sensitization. Over 13% of $CD8^+$ cells recognized this gp100 peptide after two *in vitro* sensitizations, representing an ~60-fold increase from the proportion of $tetramer^+$ cells in the *ex vivo* sample.

Th1 Responses Induced by Modified Tetanus Helper Peptide. We included a helper peptide from tetanus in this vaccine, which we modified to improve its stability. An NH_2-terminal alanine residue was added to prevent cyclization of the NH_2-terminal glutamine residue in $Tet_{830\ 844}$. The resulting 16-mer peptide AQYIKANSKFIGITEL (Tet_{A830}) has been incorporated in this Phase I trial, either as a mixture with the gp100 peptide or as part of a fusion peptide incorporating both the gp100 and tetanus peptide as a single molecule. PBLs from patients on this trial were collected before immunization, after three and after six immunizations. Samples from 20 patients were evaluated for their ability to proliferate upon the addition of the tetanus peptide directly to the PBLs (without any additional *ex vivo* sensitization). Peptide-specific proliferation was determined as a difference between [^{3}H]thymidine incorporation in experimental wells and the highest negative control (nothing or bovine albumin). We defined a SI as a ratio of maximal [^{3}H]thymidine incorporation in response to Tet_{A830} to the preimmunization value. Examples are shown in Fig. 5. A SI of 4 or greater is considered positive. We found that 3 of 5 patients (60%) in the group treated with the mixture of peptides and 6 of 7 patients (86%) in the group treated with chimeric fusion peptide responded to the tetanus peptide as a result of immunization (total, 75%; Table 5). In contrast, a response to the tetanus peptide was detected in only 1 of 8 patients vaccinated with $gp100_{280}$ peptide alone. These data, summarized in

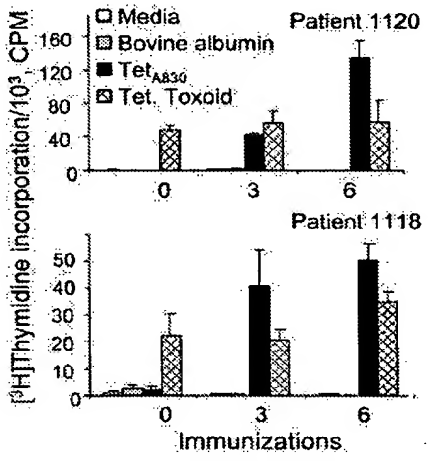

Fig. 5 Proliferative responses to tetanus helper peptide. PBLs of patients on the Mel16 trial were incubated with Tet_{A830} peptide for 5 days and then assayed for proliferation as measured by uptake of tritiated thymidine. PBLs were evaluated from prior to study entry (0 immunizations), 1 month after three vaccines (3 immunizations), and 3 months after 6 vaccines (6 immunizations). Representative data are shown for patient 1120 (*top*), who was vaccinated with the gp100 peptide and the tetanus helper peptide in Montanide ISA-51 adjuvant and for patient 1118 (*bottom*), who was vaccinated with the chimeric peptide gp100/tetanus, in QS-21 adjuvant. Responses are shown to medium alone, to bovine albumin as a negative control, to Tet_{A830} peptide, and to tetanus toxoid as a positive control. *Bars*, SD.

Table 5 Proliferative response of $CD4^+$ PBLs to Tet_{A830} peptide

SI values for all patients evaluated are shown in the table. Positive responses are those with a SI ≥4. The mean increases in reactivity were 3-, 108-, and 21-fold for the three groups. The results from patients with CTL responses to the gp100 peptide are shown in bold and were: patient 1120, who had a strong helper response; patient 1108, who had no helper response but was not vaccinated with the helper peptide; and patient 1105, who also was not vaccinated with the helper peptide.

Vaccinating peptide and adjuvant	SI		% of positive response to Tet_{A830}
	Positive	Negative	
$gp100_{280-288}$			
Montanide ISA-51		**3.6**; 3; 2; 1; 1	13
QS-21	14	1; 0	
Mixture of $gp100_{280}$ and Tet_{A830}			
Montanide ISA-51	**403**; 118		60
QS-21	16	1; 0	
Chimera $gp100_{280}$–Tet_{A830}			
Montanide ISA-51	**48**; 25	1	86
QS-21	31; 26; 10; 8		

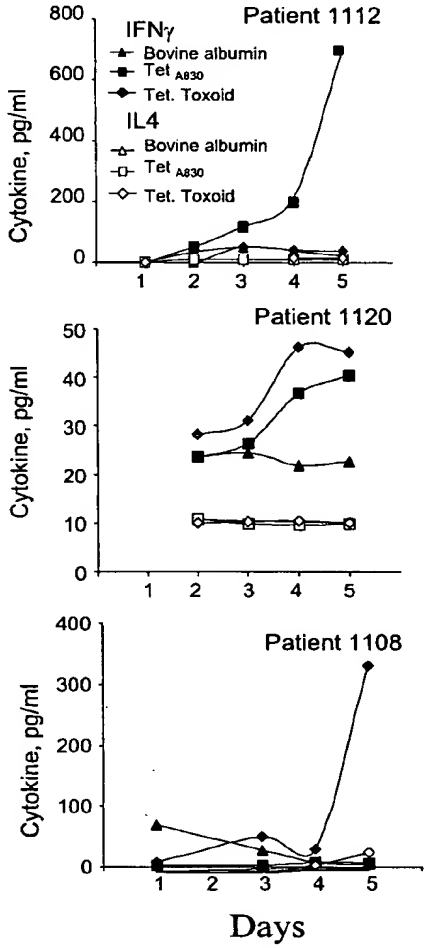

Fig. 6 Th1 cytokine secretion in $CD4^+$ T cells responding to tetanus peptide. PBLs, enriched for $CD4^+$ cells by CD8 depletion, were plated in the presence of bovine albumin (negative control), Tet_{A830}, or tetanus toxoid. Supernatants were collected daily for 5 days and were evaluated for IFN-γ, IL-4, and IL-10. There was no detectable IL-10; therefore, these data are not shown. Data for IFN-γ and IL-4 secretion are plotted for PBL samples from 2 patients (1112 and 1120) who had proliferative responses to Tet_{A830} and for PBLs from 1 patient (1108) who was not vaccinated with Tet_{A830}, as a negative control. Positive controls were assessed for all three cytokines, using phorbol 12-myristate 13-acetate and ionomycin (not shown).

Table 5, demonstrate that the modified tetanus helper peptide AQYIKANSKFIGITEL is immunogenic in humans when administered with melanoma peptides.

To assess the phenotype of the T-helper responses induced to the tetanus peptide, we evaluated cytokines produced by postvaccination PBLs from 5 patients with proliferative responses to Tet_{A830}. In each case, IFN-γ was secreted in response to Tet_{A830}, but neither IL-4 nor IL-10 was detected. Two examples are shown (Fig. 6). Thus, vaccination with Tet_{A830} helper peptide appears to induce Th1-type helper T-cell responses. The impact of these helper T-cell responses on the generation of CTLs cannot be assessed in a meaningful way in this small study.

DTH Responses and Skin Test Recall Responses. All patients were tested for reactivity to standard skin test recall antigens, and reactivity was evaluated at 24 and 48 h. Any tests with induration of at least 10 mm at 24 or 48 h are considered positive. At study entry, reactivity was observed to at least one

of these recall antigens in 18 of 22 patients (82%), including trichophyton (36%), tetanus toxoid (73%), Candida (41%), and PPD (9%). After 6–12 months, 16 patients were retested with positive reactions observed in a similar percentage of patients (50, 69, 38, and 13%, respectively). There was significant inconsistency in reactivity before and after study entry. Considering all four antigens together in the 16 patients ($n = 64$) evaluated both prior to vaccination and after study entry, reactivity was observed only prevaccine in 11 cases, only postvaccine in 12 cases, both prevaccine and after study entry in 15 cases, and absent at both time points in 26. Among the 3 patients who developed CTL reactivity to the gp100 peptide, two had reactivity to one or more recall antigens, whereas one did not.

DTH responses to the $gp100_{280}$ peptide were observed in 4 patients but did not correlate with CTL reactivity measured *in vitro* in the same patients. Two patients without *in vitro* reactivity to the peptide developed DTH responses after vaccination (patients 1104 and 1111). One patient had reactivity prevaccination and then had none after vaccination, despite evidence of CTL reactivity *in vitro* (patient 1105), and 1 patient had reactivity prevaccine but was not evaluated subsequently because she was taken off protocol for disease progression (patient 1116).

Evaluation of Adjuvants QS-21 and Montanide ISA-51. Responses to the helper peptide were observed in four of five patients (80%) who were vaccinated with that peptide in Montanide adjuvant and in five of seven patients (71%) who were vaccinated with that peptide in QS-21 adjuvant (Table 5). The mean SI was 119 for patients vaccinated with Montanide ISA-51 adjuvant and 13 for the patients vaccinated with QS-21 adjuvant. The SI is numerically higher for the Montanide patients, but in this small sample size, the difference is not statistically significant by a two-tailed t test ($P > 0.18$).

Among the 3 patients with CTL responses, 2 were vaccinated with the Montanide ISA-51 adjuvant (patient 1108, group 2; and patient 1120, group 4), and 1 was vaccinated with the QS-21 adjuvant (patient 1105, group 1). Thus, there is no meaningful difference, and each adjuvant was associated with at least one CTL response to the gp100 peptide.

Clinical Outcome. The Kaplan-Meier estimates of survival and disease-free survival for patients in this trial, from the date of protocol entry, is 86% at 1 year (95% CI, 72–100%), and 75% beyond 1.6 years (95% CI, 56.6–94.4%), with continued follow-up to 4.7 years, without additional mortality (data not shown). Disease-free survival estimates at 1 and 2 years are 68% (95% CI, 48.7–87.6%) and 57% (95% CI, 34.8–78.5%), respectively. The estimate at 2.5 years and beyond is 49% (95% CI, 24.8–72.3%), with continued follow-up to 4.7 years, without additional progression. The survival and disease-free survival estimates for patients in this trial compare favorably with outcomes reported for patients who received high-dose IFN-α (HDI) on Eastern Cooperative Oncology Group trials E1684 and E1690, in which 4-year survival rates were approximately 49 and 55%, respectively (24, 25). Patients who remain alive without disease include 3 followed >4 years, 2 followed >3 years, and 3 followed >2 years. For the 15 patients who completed the 12-month vaccine protocol without clinical evidence of disease, 100% remain alive, and 73% have been free of recurrence. All four patients who had recurrences had isolated solitary metastases [cutaneous, lymph node ($n = 2$), and liver] that were resectable, and all remain clinically free of disease (at 5+, 14+, 23+, and 35+ months).

DISCUSSION

In this Phase I trial of peptide vaccination with the $gp100_{280\ 288}$ peptide and a modified tetanus helper peptide, we evaluated toxicity and cellular immune responses. The findings are that the toxicity was limited to mild (grade I/II) local toxicity and mild (grade I/II) systemic toxicity in the vast majority of patients. All toxicities were transient. The toxicities were more prevalent for the QS-21 adjuvant than for the Montanide ISA-51 adjuvant but were not increased by the addition of the tetanus helper peptide. The toxicities were acceptable for both adjuvants and for all three peptides evaluated for the patients enrolled in this study.

The study was designed to test also for any evidence of autoimmune reactivity against normal melanocytes or normal retinal pigment epithelium. Vitiligo was not a convincing part of the patient outcome, but there was some very mild depigmentation in 2 patients that appeared to increase mildly during vaccination. There was no evidence of injury to retinal pigment epithelium either as manifested in visual acuity changes or as detected by detailed fundoscopic examination. Thus, vaccination with the gp100 peptide with or without a tetanus helper peptide, in Montanide ISA-51 or QS-21, is well tolerated with minimal negative impact on quality of life. This type of vaccine approach is markedly less toxic than what has been reported with high-dose IFN-α (24, 26–28).

The biological intent of vaccination with the $gp100_{280}$ peptide was to induce gp100-reactive CTLs in these patients. We detected CTL responses in PBLs of 3 of 21 evaluable patients (14%). These responses were not detectable in *ex vivo* samples but required sensitization *in vitro* with peptide before the responses were evident. Regardless, they were absent prior to vaccination and were increased after several vaccinations. Furthermore, the ELISPOT data were corroborated by measures of IFN-γ released directly into the medium. Thus, this is convincing evidence that vaccination with the $gp100_{280}$ peptide in adjuvant does induce T-cell responses that are detectable in the peripheral blood of some patients.

The low frequency and magnitude of CTL responses to $gp100_{280}$ peptide may be attributable to any of several factors, including the level of immunogenicity of this gp100 peptide, peptide stability, the total adjuvant effect, preexisting tolerance in patients with melanoma, or low sensitivity for detection of responses. The naturally processed $gp100_{280}$ peptide induces CTL responses naturally in some patients with melanoma, and our experience is that CTLs reactive to this peptide can be induced from most HLA-$A2^+$ melanoma patients by repeated *in vitro* stimulation of lymphocytes with HLA-$A2^+$ $gp100^+$ melanoma cells (2). Thus, there is ample evidence that immune responses to this peptide can occur when the antigen is presented effectively. When vaccinating with peptide, the intent is for the peptide to bind directly to the relevant class I MHC molecule on antigen-presenting cells in the skin, where the most relevant cells are epidermal and dermal dendritic cells (Langerhans cells). In this trial, we vaccinated into the s.c. tissue. Short

peptides, including $gp100_{280}$, have a very short half-life in fresh human plasma, suggesting that naturally occurring peptidases in the skin and plasma of humans will degrade them very rapidly (29). Migration of dendritic cells to sites of inflammation in response to the adjuvant may take several hours, and the half-life of this peptide *in vivo* may be shorter than that required for dendritic cells accumulation. Vaccination with peptide in adjuvant may be more effective when administered intradermally.

Another possible explanation for the low rate and magnitude of CTL responses to the $gp100_{280}$ peptide may be related to the approaches used for immune monitoring. In animal models of CTL responses to viral vaccines or viral infection, the peak time for CTL reactivity in the spleen is near the end of the first week after vaccination (30). This peak reactivity falls rapidly over the following weeks, leaving a small population of memory T-cells, which will proliferate promptly upon restimulation. In the present study, we drew blood for CTL assays just prior to each vaccine, which was 1–3 months after the prior vaccine. Thus, we suspect that the acute CTL response to vaccination likely peaked at 1 week and may have fallen to a much lower level by the time we had drawn blood in many of the patients. There were two patients with reactivity to the gp100 peptide by DTH, and these patients did not have evidence of CTL reactivity *in vitro*. Comprehensive assessment of CTL responses to vaccination may require more intensive evaluations of multiple lymphoid compartments, rather than evaluation of the blood lymphocytes alone.

Other studies have also detected low responses to vaccinating peptides (31, 32). Thus, the finding in the present study of CTL reactivity to the $gp100_{280}$ peptide in 14% of patients likely underestimates both the frequency and magnitude of responses. For future studies, we believe it will be more accurate to measure CTL responses in PBLs at 1-week intervals and to measure CTL responses also in a lymph node draining a vaccine site and in tumor deposits when available.

In one patient, T cells recognizing the $gp100_{280}$ peptide were detectable in a tumor-involved node and a tumor-free node, directly *ex vivo* by use of MHC-peptide tetramers. Thus, vaccination with that peptide may have induced a CTL response that persisted long after completion of the vaccine protocol (Fig. 3). T cells recognizing another HLA-A2-restricted epitope ($tyrosinase_{368\ 376D}$) peptide were not found. Despite the presence of T-cells recognizing melanoma antigens, melanoma recurred in lymph node tissue, and we are interested in determining the mechanisms that permitted immune escape by these tumor cells.

Immunohistochemical evaluation of the lymph node metastasis suggests that the tumor cells may have partially down-regulated gp100 expression, compared with the primary melanoma. This is unlikely to explain immune escape completely. It is illustrative that stimulation of the lymphocytes with tumor *in vitro*, in IL-2, does not lead to generation of $gp100_{280}$-reactive CTLs, suggesting that the tumor cells in this metastasis may not present that peptide, despite expression of gp100. A possible explanation would be a defect in antigen processing or MHC class I molecule down-regulation.

This patient also developed cutaneous lupus at the same time that she developed this tumor recurrence, presenting with a rash in the same body region. Lupus is associated with spontaneous secretion of IL-10 by circulating lymphocytes and with cellular immune dysfunction (33, 34). Furthermore, IL-10 blockade can restore cellular immune function of lupus patients (34, 35). The peripheral blood and node of this patient contained significant numbers of IL-10-secreting cells, which may well have an impact on cellular immune function as well as on the activation state of $gp100_{280}$-specific CTLs, as manifested by their inability to produce IFN-γ.

The fact that this was an isolated recurrence and that the patient has remained disease free since that time may represent some immunological control of tumor progression. It is interesting that all of the recurrences observed in patients who completed the protocol (at least six vaccines) have been isolated recurrences that were amenable to surgical resection, whereas most patients with melanoma progress in multiple sites, as did the patients who progressed early on this protocol.

The tetanus helper peptide AQYIKANSKFIGITEL has been modified to stabilize the glutamine residue at the NH_2 terminus by addition of the new NH_2-terminal alanine (A) residue. To our knowledge, this modified form of the peptide has not been used in other trials. The trial was designed such that patients were randomized to receive the tetanus peptide or not to receive it. As shown in Table 5, proliferative responses to the tetanus peptide were obtained in 86% of patients who received the chimeric fusion peptide $gp100_{280}$-Tet_{A830} and in 60% of those who received the gp100 and tetanus peptides as separate species. IFN-γ was produced by PBLs in response to the tetanus peptide, but IL-4 and IL-10 were not detectable. Thus, this modified tetanus peptide appears to induce Th1-type helper T-cell responses reliably in the majority of patients.

The patients on this trial were skin-tested with standard recall antigens, including tetanus toxoid, but this did not result in increases in T-helper responses to tetanus toxoid after vaccination. Instead, the responses to the tetanus toxoid protein remained fairly constant during the vaccine regimen (Fig. 5). Interestingly the response to the tetanus peptide Tet_{A830} does not correlate with the response to tetanus protein, suggesting that this peptide may not represent the dominant epitope from tetanus toxoid or may not be processed and presented in all HLA-DR settings. Regardless, helper T-cell responses to this peptide cannot be explained simply by changes in reactivity to tetanus toxoid but appear to be induced by vaccination with the peptide.

In summary, vaccination with an HLA-A2-restricted YLEPGPVTA peptide ($gp100_{280}$) induced CTL responses to that peptide that were detectable in 14% of patients. We believe that evaluation of PBLs only and collection of PBLs 1 month or more after each vaccine diminished the sensitivity of this assessment. We conclude that this peptide can induce CTL responses *in vivo*, but that neither the route of administration nor the approach to immune monitoring has been optimized. Tumor-infiltrating lymphocytes reactive to $gp100_{280}$ were identified in a metastatic tumor deposit that arose after vaccination, suggesting that CTLs responding to $gp100_{280}$ were induced by vaccination and are capable of migrating to tumor deposits and may persist long-term. However, in this patient, the CTLs recognizing that peptide were dysfunctional *in vivo*. Other studies have identified CTL in patients that specifically recognize a defined tumor antigen but that fail to respond appropriately to the antigen, suggesting tolerance induction or anergy (36, 37). Ad-

ditional work is needed to understand the range of etiological factors for these immune escape mechanisms.

Some patients treated on this protocol have had recurrences, and some of those patients have died with progressive metastatic melanoma, but the overall survival of patients on this protocol is excellent and compares favorably with the outcome of similar-stage patients treated with high-dose IFN-α, based on two recent Eastern Cooperative Oncology Group studies. This peptide should be evaluated further in more aggressive vaccination regimens and with more comprehensive monitoring approaches.

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